REMARKS

In view of the above amendments and the following remarks, reconsideration of the outstanding office action is respectfully requested. Pursuant to 37 CFR § 1.121, attached as Appendix A is a Version With Markings to Show Changes Made.

Angiogenesis is a precisely regulated process which coordinates the assembly and differentiation of numerous cell types to form the arteries, capillaries and veins of the pre-existing vascular bed. The primitive vasculature is composed of an endothelial plexus, which require the recruitment of pericytes and vascular smooth muscle cells by soluble growth factors secreted by endothelial cells to pattern the vessels into arteries and veins. In the final steps of vessel formation, the newly formed endothelial cells are stabilized by the extracellular matrix, the formation of a basement membrane and ensheathment with pericytes and smooth muscle cells. Numerous polypeptide growth factors have been implicated in initiating vasculogenesis and angiogenic sprouting, including fibroblast growth factors (bFGF and FGF-2), vascular endothelial growth factor (VEGF), and the angiopoietins. In addition, platelet derived growth factor B (PDGF-BB), angiopoietin-1 (ang-1), ephrin B2, and TGFβ have been shown to regulate later aspects of the angiogenesis process, in the recruitment of mural cells, and in the patterning of the vascular bed. Very little is known about growth factors which regulate the stabilization and survival of the mature vasculature, although angiopoietin-1 has been proposed as a candidate molecule. Of these factors, only VEGF has been rigorously tested for its ability to initiate angiogenesis in adults in preclinical and clinical trials. Although delivery of VEGF by gene transfer can induce an angiogenic response in ischemic tissues, exogenous VEGF induces the formation of fragile, dilated and malformed vessels. In addition, recent studies suggest that the endothelial cells of postnatal vessels may become independent of VEGF for their continued survival within several weeks of birth in rodents. Thus, the ultimate endpoint is the definition of the cellular steps and molecular sequences that direct and maintain microvascular assembly leading to therapeutic targets for repair and adaptive remodeling.

In recent studies, the roles of the neurotrophins in regulating cardiovascular development and modulating the vascular response to injury have been investigated. The neurotrophins today consist of a family of five related polypeptide growth factors: nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and neurotrophins 3, 4 (also referred to as neurotrophin 5), and 6 (NT-3 ,NT-4, NT-6). These structurally related proteins mediate their actions on responsive neurons by binding to two classes of cell surface receptor.

The low affinity neurotrophin receptor, p75, binds all neurotrophins and modulates signaling initiated by the second class of neurotrophin receptors, the trk family of receptor tyrosine kinases (what was originally identified as the trk tyrosine kinase receptor is now referred to as trk A, one member of the trk family of receptors) . Trk A, trk B, and trk C tyrosine kinases serve as the receptors for NGF, BDNF, and NT-3, respectively, and trk B can also be activated by NT-4.

NT-3 initiates a number of trophic effects on neurons expressing its receptor, trk C, ranging from mitogenesis, promotion of survival, or differentiation, depending on the developmental stage of the target cells. The reported sites of action of NT-3 reside primarily in the peripheral nervous system (PNS), various areas of the central nervous system (CNS), and in the enteric nervous system (ENS). Analyses of the phenotypes of transgenic mice lacking NT-3 or injection of embryos with a blocking antibody have revealed the essential role of NT-3 in development of specific populations of the PNS, and in particular of proprioceptive, nodose, and auditory sensory neurons and of sympathetic neurons. The actions of NT-3 also extend to modulation of transmitter release at several types of synapses in the periphery as well as in the adult CNS.

NT-4 acts via the trk B receptor and supports survival of primary somatic and visceral sensory neurons. The major visceral sensory population, the nodose-petrosal ganglion complex (NPG), requires BDNF and NT-4 for survival of a full complement of neurons, however, only one functional NT-4 allele is required to support survival of all NT-4-dependent neurons. NT-4 appears to have the unique requirement of binding to p75 for efficient signaling and retrograde transport in neurons. In addition, while all other neurotrophin knock-outs have proven lethal during early postnatal development, mice deficient in NT-4 have so far only shown minor cellular deficits and develop normally to adulthood.

Trk B receptors and BDNF are highly expressed by central and peripheral neurons, and gene ablation studies have demonstrated the critical role of trk B and BDNF in neuronal differentiation and survival, with gene targeted animals exhibiting abnormalities in cerebellar function and respiratory drive.

However, the BDNF:trk B receptor system is expressed at high levels in nonneuronal tissues, including muscle, lung, kidney, heart and the vasculature, where its biological functions are unclear. Prior studies have identified roles for the related neurotrophin, NT-3, and its receptor, trk C, in regulating cardiac septation and

valvulogenesis. In addition, it has been demonstrated that BDNF and trk B are expressed by vascular smooth muscle cells of the adult aorta, and expression of this ligand:receptor system is upregulated in neointimal cells following vascular injury. However, the biological actions of BDNF and related neurotrophins in cardiovascular function and development have not been assessed.

The present invention is directed to functions of the neurotrophins and the trk receptor family related to vascular biology.

Support for new claim 55 is found at page 17, lines 16-21 of the specification.

The objections to the specification are respectfully traversed in view of the above amendments.

The rejection of claims 7-10, 14-16, 18-19, 26-27, and 29-30 under 35 U.S.C. § 102(e) as being anticipated by U.S. Patent No. 5,733,871 to Alps et al. ("Alps") is respectfully traversed.

It is the position of the U.S. Patent and Trademark Office ("PTO") that Alps discloses administration of the claimed compounds to the same population of patients as claimed, with the same route of delivery as claimed, and inherently such compounds would effectively induce angiogenesis or promote vessel growth or vessel stabilization. Applicants respectfully disagree.

Alps relates to the treatment of neuronal damage in the central nervous system of individuals in need of such treatment. In particular, Alps relates to intravenous administration and pharmaceutically acceptable compositions of neurotrophic factors, such as bFGF, aFGF, NGF, CNTF, BDNF, NT3, NT4, IGF-I, and IGF-II, for treating or preventing neuronal damage associated with ischemia, hypoxia, or neurodegeneration. Thus, Alps relates to administration of neurotrophic factors which target neurons to improve survival and limit damage (see column 5, lines 36-41). Moreover, the examples in Alps relate to quantification of neuronal damage. More specifically, in the examples of Alps, focal or global ischemia models are used to induce neuronal damage. The efficacy of bFGF, NGF, and CNTF in treatment of the resulting neuronal damage is then analyzed. At column 9, line 9 to column 10, line 10, Alps summarizes the examples and states that bFGF reduces neuronal damage caused by ischemia.

In contrast, claim 7 (and its dependent claims 8-19 and 55) relates to "[a] method for treating a pathological disorder in a patient comprising: administering a trk receptor ligand in an amount effective to treat the pathological disorder by inducing

angiogenesis" and claim 26 (and its dependent claims 27, 29, and 30) relates to "[a] method for treating a pathological disorder in a patient comprising: administering a trk receptor ligand in an amount effective to treat the pathological disorder by promoting vessel growth or stabilization." Thus, claims 7 and 26 and their dependent claims relate to the induction of angiogenesis or vessel growth or stabilization to treat a pathological disorder. In contrast, Alps relates to the treatment of neuronal damage, which may be caused by disorders such as ischemia, hypoxia, or neurodegeneration due to stroke or cardiac arrest.

Although Alps teaches using bFGF, which it is said might also reduce ischemic damage through its trophic effects on brain glial cells and blood vessels, bFGF is not a trk receptor ligand. In particular, as set forth in Figure 3, panel B of Kaplan et al., "Tyrosine Phosphorylation and Tyrosine Kinase Activity of the *trk* Proto-Oncogene Product Induced by NGF," Nature, 350:158-160 (1991) (copy attached as Exhibit A), bFGF fails to induce Trk tyrosine phosphorylation in PC12 cells treated with 100 ng per ml bFGF. Thus, Alps neither discloses nor suggests treating a pathological disorder by inducing angiogenesis or promoting vessel growth or stabilization through administration of a trk receptor ligand.

Accordingly, the rejection based on Alps is improper and should be withdrawn.

In view of all of the foregoing, applicants submit that this case is in condition for allowance and such allowance is earnestly solicited.

Respectfully submitted,

Date: March 14, 2003

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I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: U.S. Patent and Trademark Office P.O. BOX 2327 Arlington, VA 22202, on the date below.

Date 3/14/03 | Peggy Dimyer

Appendix A

Version With Markings to Show Changes Made

In reference to the amendments made herein to the specification, additions appear as underlined text, while deletions appear as bracketed text, as indicated below:

In The Specification:

At page 1, lines 3-5:

The present application claims the benefit of U.S. Provisional Patent Application Serial No. 60/105,928, filed October 28, 1998, now abandoned, and U.S. Provisional Patent Application Serial No. 60/119,994, filed February 12, 1999, now abandoned.

At page 9, line 16 to page 10, line 3:

Figures [6A-I] 6A-G show that BDNF supports the survival of cardiac microvascular endothelial cells. Panels A and B show flow cytometric analysis of cardiac microvascular endothelial cells incubated with anti-CD31 antisera (panel A) or control IgG (panel B). 97% of cells exhibit CD31 reactivity and 1% react with control IgG. Panel C shows RT-PCR analysis of transcripts for BDNF and kinase active trk B mRNA in cardiac microvascular endothelial cells ("ECs") and adult murine brain (B). Amplification of BDNF (360 bp) and the regions of the kinase domain of trk B (571 bp) are detectable in cardiac endothelial cells and adult brain samples. To ensure the absence of DNA contamination, RNA samples were amplified using primers without the reverse transcription step and these reactions yielded no products (no RT). Panels D, E, and F show TUNEL analysis of microvascular endothelial cells. ECs were cultured in media containing 10% serum (panel D), or in media containing 0% serum (panels E and F) in the presence of BDNF (100ng/ml) (panel F) for 48 hours. 1500 cells per sample were analyzed and the mean and standard deviation of four samples is indicated. Results are representative of two experiments performed in quadruplicate on cultures from different litters of animals (Magnification: 20X). Panel G shows flow cytometric analysis of annexin V binding. Cardiac microvascular endothelial cells were cultured in the indicated conditions in the presence of BDNF (25ng/ml)

or VEGF (10ng/ml) for 48 hours prior to incubation with FITC-annexin V and propidium iodide for flow cytometry of 1 x 10^4 cells per condition. Results are representative of two independent experiments performed on cultures from different litters of animals.

Tyrosine phosphorylation and tyrosine kinase activity of the *trk* proto-oncogene product induced by NGF

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NERVE growth factor (NGF) is a neurotrophic factor responsible for the differentiation and survival of sympathetic and sensory neurons as well as selective populations of cholinergic neurons[1,2]. NGF binds to specific cell-surface receptors but the mechanism for transduction of the neurotrophic signal is unknown. Several experiments using the NGF-responsive pheochromocytoma cell line, PC12, have implicated tyrosine phosphorylation in NGF-mediated responses, although no NGF-specific tyrosine kinases have been identified[3]. Here we show that NGF induces tyrosine phosphorylation and tyrosine kinase activity of the *trk* proto-oncogene product, a tyrosine kinase receptor whose expression is restricted *in vivo* to neurons of the sensory spinal and cranial ganglia of neural crest origin[4]. Tyrosine phosphorylation of *trk* by NGF is rapid, specific and occurs with picomolar quantities of factor, indicating that the response is mediated by physiological amounts of NGF. Activation of the *trk* tyrosine kinase receptor provides a possible mechanism for signal transduction by NGF.

In the presence of NGF, PC12 cells differentiate into sympathetic neurons, extend neurites, increase neurotransmitter synthesis and become electrically excitable[5-7]. Several unidentified proteins are phosphorylated on tyrosine in these cells and the introduction of the tyrosine kinase v-*src* into PC12 cells stimulates an outgrowth of neurites similar to that resulting from NGF treatment[3,8]. Therefore, events mediated by tyrosine phosphorylation could be involved in the differentiation of PC12 cells.

The *trk* gene is a member of a gene family of tyrosine kinase (TK) receptors that includes the related gene *trk*b[4,9,10]. To determine if *trk* or *trk*b were transcribed in PC12 cells, the expression of *trk* transcripts was assayed by northern-blot transfer analysis with a full-length *trk* complementary DNA probe or with an extracellular domain, *trk*b-specific probe[9]. PC12 cells expressed a 3.2-kilobase *trk* transcript, but *trk*b transcripts were not detected (Fig. 1*a*). The level of *trk* transcripts was not affected by the addition of NGF. To determine whether additional *trk*-related genes were transcribed in PC12 cells, messenger RNA was hybridized at low stringency with the highly conserved *trk* TK domain. No additional *trk*-hybridizing transcripts were detected (not shown).

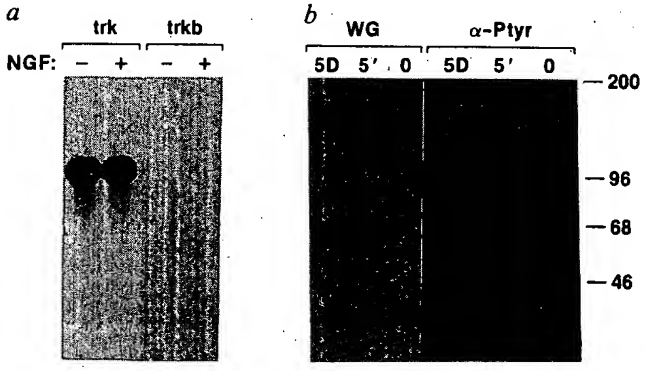

FIG. 1 *a*, Northern-blot transfer analysis of *trk* and *trk*b transcripts in NGF treated (+) or untreated (−) PC12 cells. *b*, Ptyr: phosphotyrosine-containing proteins in PC12 cells treated for 5 min or 5 days with NGF or in untreated cells (0). WG, tyrosine-phosphorylated glycoproteins in cells treated with NGF for 5 min or 5 days. *M*, markers (in K) are indicated.
METHODS. *a*, RNA preparation and northern blot transfer analysis was performed as described previously[4]. Cells were treated with 50 ng per ml NGF(+) (Boehringer-Mannheim) and were collected 48 h later after differentiation had occurred. Total RNA (20 μg) was loaded per lane and the filter was hybridized with a probe specific for *trk*[4] or *trk*b[9]. *b*, Ptyr; PC12 cells were treated with 50 ng per ml NGF for 5 min or 5 days. Cells were washed, lysed and the lysates (50 μg protein per lane) analysed by immunoblotting with the phosphotyrosine (Ptyr) monoclonal antibody 4G10 (gift from D. Morrison, B. Drucker and T. M. Roberts) as previously described[17,18]. For WG, lysates (prepared from 2×10^7 cells) were incubated with 100 μl of a 50% solution of wheat-germ (WG) lectin agarose (Pharmacia) for 2 h at 4 °C. Precipitates were washed and analysed by western blot analysis with anti-Ptyr as previously described[17,18]. Electrophoresis was performed on 7.5% SDS-polyacrylamide gels.

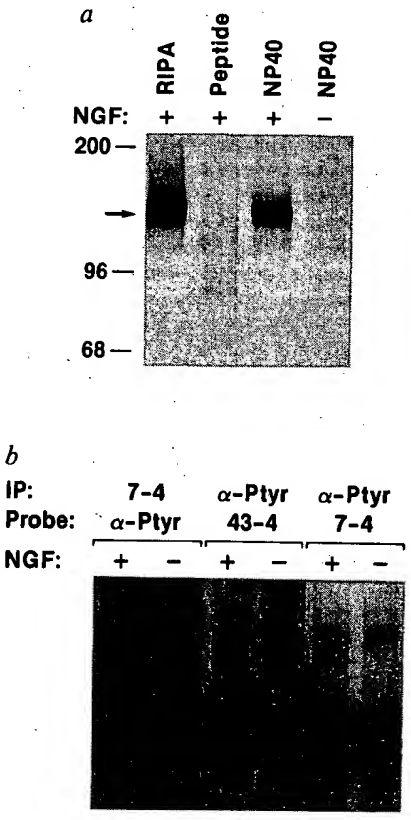

FIG. 2 NGF induces tyrosine phosphorylation of Trk. *a*, Trk was immunoprecipitated with rabbit peptide antibody 43-4[11] from lysates of NGF-treated (+) or untreated (−) PC12 cells and the immunoprecipitates were probed with anti-Ptyr. Cells were lysed in buffer containing 1% NP40[18] or RIPA buffer[19]. Immunoprecipitations were performed in the presence or absence of competing peptide (10 μg ml^{-1}). Similar results were obtained with other sources of NGF (Collaborative Research, Upstate Biotechnology Inc.). *b*, NGF-induced tyrosine phosphorylation of Trk in anti-Trk or anti-Ptyr immunoprecipitates. Trk immunoprecipitates were prepared with rabbit antibody 7-4[11] before anti-Ptyr western blot analysis or with Ptyr antibody before western blot analysis with Trk antibodies 43-4 or 7-4.
METHODS. PC12 cells were treated for 5 min at 37 °C with 50 ng per ml NGF. Cells (2×10^7) were lysed in buffer containing 1% Nonidet P-40 (NP40) (except as noted) and were immunoprecipitated with anti-Trk or anti-Ptyr for 2–3 h at 4 °C as described previously[18]. Immunoprecipitates were collected with protein-A/Sepharose, subjected to 7.5% SDS-PAGE and analysed by immunoblotting as described[18]. *M*, markers (in K) are indicated.

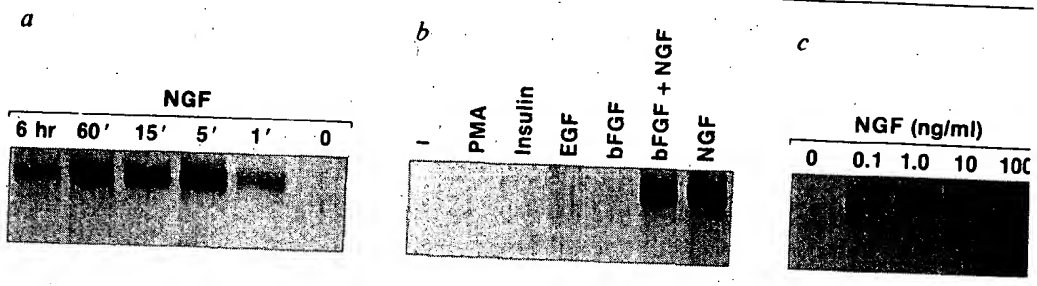

FIG. 3 Time course, growth factor specificity and dose-response of Trk tyrosine phosphorylation in PC12 cells. *a*, Time course of Trk tyrosine phosphorylation. Cells (2×10^7) were treated with 50 ng per ml NGF at 37 °C. *b*, Effects of growth and differentiation factors on Trk tyrosine phosphorylation. Cells were treated with 100 ng per ml NGF, 100 ng per ml basic fibroblast growth factor (bFGF) (Boehringer–Mannheim), 100 ng per ml epidermal growth factor (EGF) (Upstate Biotechnology, Inc.), 100 nM insulin (Sigma), or 1 μg per ml phorbol 12-myristate 13-acetate (PMA) (Sigma) for 5 min at 37 °C. *c*, Dose-response of Trk tyrosine phosphorylation. Cells were treated for 30 min at 37 °C with increasing concentrations of NGF. Western blot analysis with Ptyr antibodies of Trk immunoprecipitates prepared with anti-Trk antibody 43-4 are shown.

Phosphotyrosine (Ptyr) western-blot transfers of cell lysates prepared from PC12 cells treated with NGF for 5 min demonstrated the tyrosine phosphorylation of a broad band at relative molecular mass 130,000–140,000 (M_r 130–140K) (Fig. 1*b*). Proteins of 42, 45, 50, 60 and 150K were also observed to be phosphorylated on tyrosine in response to NGF treatment of PC12 cells. A band of 130–140K was observed when the cell lysates were precipitated with wheat-germ lectin before analysis by anti-Ptyr immunoblotting, indicating that NGF induced the tyrosine phosphorylation of a glycosylated protein (Fig. 1*b*).

To determine whether Trk, a 140K glycoprotein[11], was phosphorylated on tyrosine in response to NGF, Trk protein was immunoprecipitated with an antibody generated against a C-terminal Trk peptide (43-4)[11] and the immunoprecipitates were probed with anti-Ptyr antibodies. Trk was phosphorylated on tyrosine after addition of NGF to the cells (Fig. 2*a*). Immunoprecipitation of Trk was specifically blocked by a Trk-derived peptide and was not reduced by immunoprecipitation in stringent buffers such as RIPA (Fig. 2*a*). Tyrosine phosphorylation of Trk was also observed when the immunoprecipitations were performed using a second anti-Trk antibody generated against the $p70^{trk}$ oncogene product (7-4)[11] and when anti-Ptyr immunoprecipitates prepared from NGF-treated cells were probed with either of two anti-Trk antibodies (Fig. 2*b*). The same amount of Trk protein was recovered in each immunoprecipitation, as measured by [^{35}S] methionine labelling or Trk (not shown). To confirm that Trk was phosphorylated on tyrosine residues in response to NGF, PC12 cells were labelled with ^{32}P-orthophosphate before NGF addition and immunoprecipitation with anti-Trk antibodies. Phosphoamino-acid analysis demonstrated that Trk was phosphorylated on tyrosine in response to NGF (not shown).

Trk tyrosine phosphorylation occurred within 1 min of NGF treatment of cells, reached maximum levels after 5 min and declined thereafter (Fig. 3*a*). Residual phosphorylation was detected after two days of treatment with NGF when the cell population was fully differentiated (not shown). Trk tyrosine phosphorylation was also specific to NGF. Other peptide growth factors that elicit tyrosine phosphorylation in PC12 cells were tested in our assay[3,12]. Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin and the phorbol ester phorbol 12-myristate 13-acetate (PMA) failed to induce Trk tyrosine phosphorylation (Fig. 3*b*). No additional tyrosine phosphorylation of Trk was seen in cells treated with both bFGF and NGF (Fig. 3*b*). These agents produce similar patterns of early responses in PC12 cells, including transcriptional activation of c-*fos* and c-*myc*[13]. But of these factors, only NGF and bFGF stimulate neurite outgrowth.

To determine the minimal concentration of NGF required for Trk tyrosine phosphorylation, a dose-response experiment was performed. Tyrosine phosphorylation was half-maximal at 0.1 ng per ml NGF (50 pM) (Fig. 3*c*), indicating that Trk phosphorylation occurs at physiologically relevant concentrations of NGF[14].

NGF also induces the tyrosine kinase activity of Trk. Trk was phosphorylated *in vitro* in immunoprecipitates prepared from PC12 cells treated for 5 min with NGF, presumably owing to catalytic autophosphorylation (Fig. 4). No Trk phosphorylation was detected in immunoprecipitates from untreated cells or when competing Trk-derived peptide was included during immunoprecipitation.

Our results show that the protein tyrosine kinase receptor Trk is a substrate for an NGF-induced tyrosine kinase activity in PC12 cells. The rapid and specific response suggests that Trk is closely associated with the receptor for NGF. Alternatively, the Trk protein may directly bind NGF. The kinase activity of Trk

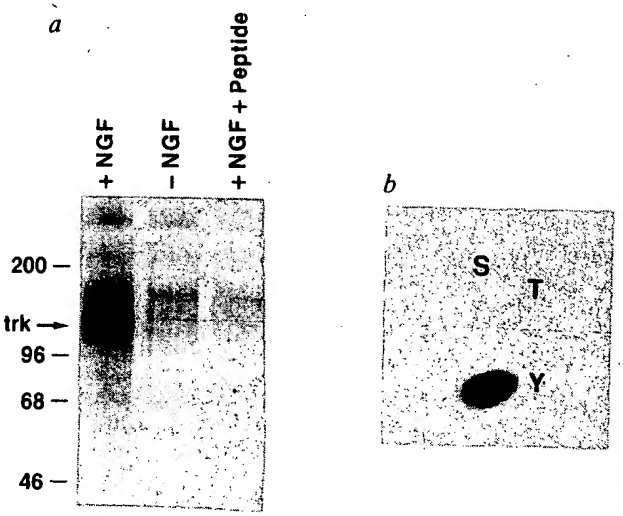

FIG. 4 Activation of Trk kinase activity by NGF. *a*, Immune-complex kinase assay following immunoprecipitation with anti-Trk antibodies prepared from NGF-treated (+) or untreated (–) PC12 cells. Immunoprecipitations were performed in the presence or absence of competing peptide (10 μg ml^{-1}). *b*, Phosphoamino-acid analysis autoradiogram of Trk phosphorylated *in vitro* in *a*. The positions of serine (S), threonine (T) and tyrosine (Y) are indicated. METHODS. PC12 cells (8×10^7) were lysed and precipitated with wheat-germ lectin–agarose (300 μl 50% solution) as for Fig. 1. The precipitates were washed three times in 1% NP40-containing lysis buffer[18] and the glycoproteins were eluted in 400 μl 0.5 M *N*-acetylglucosamine in lysis buffer containing 1 mM orthovanadate for 1 h at 4 °C. The eluate was diluted 10-fold with lysis buffer and immunoprecipitated with anti-Trk antibody as described in Fig. 2 legend. The immunoprecipitates were incubated with 20 μCi [γ-^{32}P] ATP, 10 mM $MnCl_2$, 20 mM Tris, pH 7.4, for 20 min at 25 °C. Phosphorylated proteins were analysed by 7.5% SDS-PAGE as described[18]. M_r markers (in K) are indicated. For *b*, the phosphorylated Trk band was eluted from the gel and phosphoamino-acid analysis performed as previously described[21].

may also be activated by phosphorylation of Trk by another cellular tyrosine kinase.

Trk may be responsible for the increases in phosphotyrosine in NGF-treated PC12 cells. Several proteins phosphorylated on tyrosine in these cells are candidate substrates for the Trk-induced kinase activity, including microtubule associated protein-2 (MAP-2) kinase and phospholipase C_γ[15] (M. Vetter, D.M.-Z., L.F.P., J. M. Bishop and D.R.K., manuscript submitted). Examination of Trk immunoprecipitates for co-immunoprecipitating substrates should facilitate the identification of other targets of tyrosine kinase activities in PC12 cells.

The expression *in vivo* of *trk* is consistent with its proposed role in NGF-mediated events. The *trk* mRNA is restricted to the dorsal root ganglia and cranial sensory ganglia of neural crest origin, tissues comprised primarily of neurons that are trophic substrates for NGF[4]. These neurons rely on NGF for differentiation and maintenance of the neuronal phenotype[16]. Sympathetic neurons and certain neurons in the central nervous system, which may not express *trk*, also respond to NGF[1,2]. But other *trk* family members, for example *trk*b, are expressed in the central nervous system[9,10] and may function as NGF-responsive tyrosine kinases in these tissues. Other tyrosine kinases expressed in neurons, including $p60^{c\text{-}src}$ and $p62^{c\text{-}yes}$, may be involved in NGF responses, although the phosphorylation state and kinase activity of these proteins does not seem to be altered in response to addition of NGF (M. Vetter, D.K. and M. J. Bishop, unpublished results). Trk is a likely candidate for a transducer of NGF signals. These results implicate tyrosine kinase receptors as possible mediators of neurotrophic signals in vertebrates. □

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A cellular protein that competes with SV40 T antigen for binding to the retinoblastoma gene product

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TUMOUR-suppressor genes, such as the human retinoblastoma susceptibility gene (*Rb*), are widely recognized as being vital in the control of cell growth and tumour formation[1]. This role is indicated, in part, by the suppression of tumorigenicity of human tumour cells after retrovirus-mediated *Rb* replacement[2–4]. How *Rb* acts to bring about this suppression is not clear[5] but one clue is that the Rb protein forms complexes with the transforming oncoproteins of several DNA tumour viruses[6–8], and that two regions of Rb essential for such binding frequently contain mutations in tumour cells[9,10]. These observations suggest that endogenous cellular proteins might exist that bind to the same regions of Rb and thereby mediate its function. We report here the identification of one such human cellular Rb-associated protein of relative molecular mass 46,000 (46K) (RbAP46). Two lines of evidence support the notion that RbAP46 and simian virus 40 T antigen have homologous Rb-binding properties: first, several mutated Rb proteins that failed to bind to T also did not associate with RbAP46; and second, both T antigen and T peptide (amino acids 101–118) were able to compete with RbAP46 for binding to Rb. The apparent targeting of the RbAP46–Rb interaction by oncoproteins of DNA tumour viruses strongly suggests that formation of this complex is functionally important.

Previous attempts to identify Rb-associated proteins[11] may have been hampered by insufficient quantities of such stable complexes in cells. One approach to overcome this difficulty is to add excess free Rb protein *in vitro*, thereby driving complex formation. Detectable amounts of RbAPs could then be coprecipitated by anti-Rb antibody. To obtain large quantities of Rb protein, we constructed plasmid pETRbc, containing the entire coding region of Rb, for high-level expression in *Escherichia coli*[12] (Fig. 1*a*). The most stably expressed protein was a 56K polypeptide (p56-Rb), which was then purified (Fig. 1*b*) and characterized immunologically (Fig. 1*c*). Only antibodies recognizing the C-terminal half of Rb could precipitate this protein, whereas two different antibodies recognizing the N-terminal regions failed to precipitate it. The C-terminal 56K contains both regions essential for T binding[9,10]. When purified T antigen was added to bacterial lysates, this 56K polypeptide was coprecipitated by anti-T antibody (data not shown). Conversely, mixing increasing amounts of purified 56K protein with radiolabelled COS cell lysates, in which T is expressed in great excess over endogenous Rb[7], resulted in quantitative coprecipitation of T by anti-Rb antibody over a 10-fold range (data not shown). Similar results were obtained by mixing COS cell extracts with lysates of bacteria expressing Rb protein (Fig. 3, lanes 1 and 2). These studies indicated that bacterially expressed p56-Rb retained the T-binding properties of full-length Rb.

We next tested whether a cellular protein exists in human HeLa cell extracts that binds to p56-Rb in a similar way. As shown in Fig. 2*a*, a protein of 46K (RbAP46) was specifically detected only when p56-Rb, either in bacterial lysates (Fig. 2*a*, lane 2) or in a purified form (Fig. 2*a*, lanes 4–7), was mixed with radiolabelled HeLa cell lysates. By mixing increasing amounts of purified p56-Rb with constant amounts of HeLa cell lysates, the 46K protein was immunoprecipitated in increasing amounts (Fig. 2*a*, lanes 3–7), suggesting that it formed a complex with the input 56K protein. To exclude the possibility of cross-reaction of the 46K protein with the rabbit anti-Rb 0.495, another anti-Rb antibody, 0.47, was used[10], and again the 46K protein was coprecipitated (Fig. 2*b*, lane 11). But no 46K protein was detected if nonimmune serum was used (Fig. 2*b*, lane 9). These results implied that this 46K protein was directly associated with p56-Rb. Using a full-length Rb protein expressed in insect cells[13], the same 46K protein was again coprecipitated (Fig. 2*c*, lane 15), although some additional protein bands were also seen. As RbAP46 was the major protein detected by using either a full-length Rb or p56-Rb, we concluded that p56-Rb contains all the necessary sequences for binding to the 46K protein.

The specificity of the interaction was further characterized by defining the regions of Rb involved in binding to RbAP46. Five

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